

# Modeling growth factor activity during proinflammatory stress: Methodological considerations in assessing cytokine modulation of IGF binding proteins released by cultured bovine kidney epithelial cells<sup>☆</sup>

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## Abstract

The present research was conducted to model potential mechanisms through which IGFBPs might be affected by a key proinflammatory response initiating cytokine tumor necrosis factor (TNF)- $\alpha$ . Madin–Darby bovine kidney epithelial (MDBK) cells, known to release IGFBPs in response to several stimuli, were grown under several conditions and challenged with forskolin (F) or recombinant TNF- $\alpha$  for 24 h. Forskolin increased IGFBP-3 gene expression and media content of BP-3 protein. TNF- $\alpha$  increased basal and augmented F-mediated IGFBP-3 gene expression. However, TNF- $\alpha$  effects on the measurable media content of IGFBPs were influenced by culture conditions; in the absence of added protease inhibitors (PIs) or sufficient media albumin concentration (high BSA, 1 mg/ml), the effect of TNF- $\alpha$  was to decrease ( $P < 0.02$ ) measurable IGFBPs. In the presence of PI and high BSA, media IGFBP-3 levels were shown to be increased by TNF- $\alpha$  consistent with the gene expression data. Changes in media IGFBP-3 protease activity were examined further to explain the observed effects of TNF- $\alpha$  on production and destruction of IGFBPs in media. When recombinant human IGFBP-3 (500 ng/ml) was added to PI-free, low BSA 100  $\mu$ g/ml media from TNF-treated MDBK cells, less than 10% of the BP-3 was recognizable by Western blot in 30 min; conversely, inclusion of High BSA and PI in media resulted in attenuation of the protease effect on the IGFBPs. The data suggest that the MDBK model of cellular response to proinflammatory stimulus is affected by culture conditions and that TNF- $\alpha$  affects media content of IGFBPs through effects on IGFBP gene expression coupled with degradation of IGFBPs via enhanced proteolytic enzyme release.

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## 1. Introduction

The roles for insulin-like growth factors (IGF) as metabolic modifiers have evolved beyond noted endocrine effects to encompass many localized paracrine actions affecting cell survival through antiapoptotic activity [1–3], regulation of blood flow via modulation of nitric oxide synthase activity [4,5], and modification of the intracellular redox milieu [6]. As important as

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the IGF-s are themselves, it is now well recognized that changes in the ubiquitous milieu of IGF binding proteins (IGFBPs) plays a significant role in modifying and modulating specific biochemical and physiological functions of the IGFs [7–10]. Immune stress and the accompanying proinflammatory state has been associated with decreased circulating plasma levels of IGF-1 and altered patterns of IGFBPs [11–13]. It is thought that these changes reflect the capacity to balance and realign metabolic needs with survival and the establishment of a homeostatic environment to thwart the progressive cascade toward multiple organ failure. In fact, de Groof suggested that the positive clinical outcome to severe sepsis correlated with the ability for plasma IGF-1 to be depressed and its distribution to tissues modulated through a differential effect on the pattern of binding proteins [14]. However, IGF-1 mRNA and local tissue concentrations of IGF-1 do not always correlate with measured changes in plasma IGF-1 during immunologically stressful situations [12]. The data suggest that collectively many metabolic features of the proinflammatory response are dictated by the interaction of IGF-1 with the IGFBP-3 complex at the localized cellular level to regulate sepsis-related inhibition of enzyme function [12,15,16], the proteolytic enzymes in particular.

With the onset of recognition of an immunological threat, a cascade of cytokine-driven responses is initiated. A significant first line of defense is the elaboration of initiation response cytokines such as TNF- $\alpha$  and its associated signal transduction elements such as NF- $\kappa$ B [17]. With the progression through the acute phase response, a relative balance between these initiating cytokines [18] and anti-inflammatory cytokines such as IL-10 [19] develops. Contemporary with the development of this proinflammatory cascade, metabolic changes to a more catabolic/less anabolic state, consistent with observed decreases in plasma concentrations and tissue mRNA content of IGF-1 [11,13,14,20]. Evidence exists to suggest that many of the effects on metabolism are mediated through the direct actions of the proinflammatory cytokines themselves on cells and organs via specific receptors [11,18,20,21]. Data further suggest that additional levels of IGF-related metabolic regulation develop, namely, in the capacity for IGF binding protein expression and release into the local environment to modify the distribution of IGF-1 to cells [13]. However, a direct demonstration that the effect of immune stress on IGFBPs can be directed by proinflammatory cytokines like TNF- $\alpha$  is lacking in the literature. The selective modulation of IGF binding proteins directly at the tissue level would permit differential and selective actions of IGFs to be retained while other

actions are suspended. Examples of this are suggested in the literature where antiapoptotic tissue repair is driven by a retained localized IGF-1 action downregulating NF- $\kappa$ -B catabolic pathways. Other examples are in instances where the actions of IGF-1 are modified by the localized change in transgene expression of a particular binding protein or binding protein subunit [3,22,23] while the peripheral circulating plasma concentrations of IGF-1 remain decreased. Similar paracrine effects of deliberate site-directed expression of IGF binding proteins have been shown to modulate some effects of disease states with positive outcomes [7,12,16], a critical feature considering the close interplay between the somatotrophic and immune axes during compromised health [24,25].

Unlike many other species, there are few, if any, validated *in vitro* models relevant to the bovine that have application in exploring cellular response to proinflammatory stress. The basic fragility of bovine hepatocytes which results in tremendous loss of viability, and observed laboratory-to-laboratory variability in the functional responsiveness of collagenase-isolated cells has largely hindered their use as culturable cells capable of uniformly reacting to biological response modifiers. To further address this aspect of the endocrine-immune interaction, we conducted the present experiments to define the effects of culture conditions on an *in vitro* model of proinflammatory stress. Specifically, we addressed how culture conditions affect the interpretation of the effects of TNF- $\alpha$  on the pattern of IGFBPs released into media by a line of epithelial cells of bovine origin available to researchers through the American Type Culture Collection (Manassas, VA).

## 2. Materials and methods

### 2.1. Cells, general reagents, and supplies

Experiments were performed and replicated separately in three locations: two laboratories within the Growth Biology Laboratory at the USDA (THE and CL), Beltsville and one laboratory at Auburn University (JLS) under similar conditions. Madin Darby bovine kidney epithelial cells were obtained from American Type Culture Collection (Manassas, Va.). Recombinant bovine TNF- $\alpha$  was a generous gift of Novartis Ltd. (formerly Ciba Geigy, St. Aubin, Switzerland). RPMI1640 medium, fetal bovine serum (FBS), trichloroacetic acid, trypan blue, trypsin, forskolin, bovine insulin, transferrin, protease inhibitor cocktail for mammalian cell extracts, selenium and reagents for determination of total urea nitrogen, were procured from Sigma Co. (St. Louis, MO). For poured 1%

acrylamide gels, Tris–glycine buffer components, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, bromocresol blue, and electrophoresis and semidry transfer blotting equipment were obtained from Bio-Rad, Inc. (Hercules, CA). Gradient gels (4–20%) were procured from Invitrogen Life Technologies (Carlsbad, CA). Rabbit anti-bovine IGFBP-2, recombinant human IGFBP-3 (hIGFBP-3) and rabbit anti-hIGFBP-3 were obtained from Upstate Biotech, Inc. (Lake Placid, NY). Plastic cultureware (T-25 flasks and 12-well plates) was obtained from Costar Inc. (Cambridge, MA).

## 2.2. Model characterization and optimisation

MDBK cells were grown from frozen storage in T-25 flasks in RPMI 1640 supplemented with 5% FBS, insulin (1 µg/ml), transferrin (5 µg/ml), and selenium (1 µg/ml) under atmospheric conditions of 5% CO<sub>2</sub> in air. To eliminate potential confounding effects of estrogenicity and pseudo-growth factor activity on IGFBPs and proteolytic enzyme release [26] media preparations were phenol red-free [27]. After cultures were established, cells were passed and replated three times at 1:10 dilution in RPMI or Eagle's medium supplemented with 1% FBS in the absence of added insulin, transferrin and selenium. For experimental use, cells were dislodged with standard trypsinization and replated at a seeding density of 10<sup>6</sup>/well in 12-well plates. The basic system used to study the production and release of IGF binding proteins from MDBK cells was that originally outlined by Cohick et al. [28]. Treatments were applied to cells in four wells/replicate with the individual well treatments applied in serum-free medium supplemented with 50 µg/ml heat-treated bovine albumin free of IGF binding proteins (Sigma A-7888 RIA grade BSA) and randomly assigned and distributed across several culture plates. Experiments were repeated *in toto* three times.

Noting that the morphological appearance and responsiveness of MDBK cells to hormonal stimuli changed with the time in culture and state of confluence, we assessed metabolic activity and hormone responsiveness of MDBK cells as well as the release of IGF binding protein (IGFBP-2) into culture medium at approximately 90% confluence and at later stages of dense confluence where evidence of tight junction dome formation was present. For this purpose cell culture plating was staggered so that on a given day early confluence and domed-confluence were studied in concurrent treatment applications. Media were harvested and the effect of treatments on the media content of IGFBP-2 was assessed by Western blot using rabbit anti-bovine IGFBP-2. An additional estimation of metabolic activity as affected by cytokine

and growth hormone (GH) interactions in recent confluent and domed-confluent cells was made in a complementary set of culture plates with media supplemented with 250 mM arginine [29,30], with the subsequent production of urea from arginine permitted to progress for 3 h. In these cultures, TNF-α was also added to cells at 0 or 10<sup>-7</sup> M.

For determination of IGFBP content in media, cells just reaching confluence were washed twice for 6 h/wash in serum-free medium. Cytokine and forskolin treatments were applied for 24 h at which point the media was harvested and stored frozen at -80 °C until assayed for IGFBP-2 and -3. Cell viability was assessed by standard trypan blue exclusion. Viability was greater than 90% throughout the studies regardless of cytokine or forskolin treatment, though later experiments where a protease cocktail was included in the media, the viability and attachment of cells decreased to ~70%. TNF-α and forskolin effects were assessed in a factorialized experimental arrays of concentrations (TNF-α: 0, 10<sup>-9</sup> or 10<sup>-7</sup> M; forskolin: 0, 0.1, 1 or 10 µM) as dictated by the experimental design. Data were the average of 4 wells/culture study.

<sup>125</sup>I-IGF-1 ligand blotting or Western blot were used to identify and quantify media concentration differences in IGF binding proteins. Changes in the relative media concentrations of IGFBP-2 and -3 were estimated by scanning laser densitometry of autoradiograph bands (<sup>125</sup>I ligand blotting) on X-Omat AR-5 film (KODAK, Rochester, NY) or nitrocellulose (IGFBP-2 Western blot) as generated by alkaline phosphatase staining and rabbit anti-bovine IGFBP-2 antibody used at 1:1000. For ligand and Western blotting, samples were prepared for electrophoretic protein separation under nonreducing conditions as originally described by Hossenlopp et al. [31] and further validated for IGFBP studies by Cohick et al. [28]. The general protocol for IGFBP determination was as follows: media samples were diluted 1:2 with glycerol SDS (0.1%) loading buffer containing bromocresol blue, incubated at either 60 °C for 10 min or 95 °C for 5 min and loaded onto 12% or 4–20% acrylamide gels. For each gel, 10 lanes were loaded with the media samples and 2 additional lanes loaded with a similarly prepared plasma (1.0 µl) sample to serve as an internal standard to normalize band densities across the multiple gels. Proteins were separated for 1.5 h at 110 V and transferred to nitrocellulose (0.2 µm, Bio-Rad, Hercules, CA) for ligand or antibody probing. For ligand blotting, nitrocellulose membranes were washed in Tris–saline (10 and 135 mM, respectively, pH 7.5) for 10 min containing nonionic detergent (NP-40, 1%), further washed three times in Tris–saline and

Table 1  
Gene expression probe information for IGFBP-2 and IGFBP-3

Gene	Gene bank accession number	Product BP	Primer sequence <sup>a</sup>	Annealing temperature (°C)	Cycles
IGFBP-2 (bovine)	NM.174555	175	Sense ATG GGC AAG GGT GGC AAA C; antisense TGG GGA TGT GTA GGG AGT AGA G	63.0	40
IGFBP-3 (bovine)	NM.174556	120	Sense GCA GCT GGC TAC AGC CTC AAC TTC; antisense GCC AGG TTC ACC ATG TGG AAG AAA	56.2	40
GAPDH (rat)	AF106860	87	Sense TGC ACC ACC AAC TGC TTA GC; antisense GGC ATG GAC TGT GGT CAT GAG	55.0	40

<sup>a</sup> All sense and antisense primers were synthesized by and purchased from Invitrogen, Carlsbad, CA.

blocked against nonspecific binding in the presence of 2% radioimmunoassay grade bovine serum albumin (Sigma A-7888, St. Louis, MO) and 0.1% Tween 20 for 1 h. <sup>125</sup>I-recombinant human IGF-1 (250,000 cpm, Amersham) was added to the incubation vessel (10 ml buffer) and the incubation performed overnight at 4 °C. The blots were washed three times in Tris–saline, dried and placed in association with the X-ray film at –80 °C. Band patterns and grey-scale intensities on the film were measured by densitometry. Western blot identification and measurement of IGFBP-2 was performed on similarly prepared nitrocellulose blots with identical preparation and protein blocking. The anti-IGFBP-2 was added to the incubation mixture at a 1:1000 dilution (10 ml), incubated overnight, washed three times and exposed to goat anti-rabbit IgG-conjugated to alkaline phosphatase (1:3000) using BCIP-NBT as the substrate.

### 2.3. Effects of TNF- $\alpha$ on IGFBP gene expression in MDBK cells

MDBK cells were scraped from plates and total RNA extracted using TRIzol reagent and purified using the RNeasy kit (Qiagen, Vencia, CA). RNA quality was determined spectrophotometrically and by electrophoresis. RNA (1  $\mu$ g) was reverse transcribed (Iscrip cDNA synthesis kit, BioRad, Hercules, CA). The cDNA was subjected to RT-PCR using primers designed for bovine IGFBP2 and IGFBP3 sequences (Table 1), primers for a control housekeeping gene (bovine glyceraldehyde phosphate dehydrogenase, bGAPDH), and Eppendorf TAQ DNA Polymerase (Fisher Scientific, Pittsburgh, PA). PCR products were cloned into pCRII (Invitrogen) and recombinant plasmids transformed in *E. coli* INVF competent cells. Plasmids were isolated and quantified by optical measurements at 260 nm. The PCR product sequences were determined (AU Genomics and Sequencing Lab) and sequences verified.

Temperature gradients were performed for all primer sets to determine optimum annealing temperature and

primer quality for duplicate blank, standards, and RT product for each temperature. In addition, standards or PCR products were run in 10-fold dilution series to determine an optimum range for the standard curves. Dilutions of plasmids with the gene of interest were used as standards. Sample dilutions were linear across the range of the assay.

Real time PCR was performed using a BioRad MyIQ thermocycler with a total reaction volume of 30  $\mu$ l (15  $\mu$ l of SYBR Green, 0.6  $\mu$ l of each primer and 13.8  $\mu$ l of RT plus water). A denaturation cycle of 95 °C for 30 s followed by amplification and quantification at 95 °C for 30 s, optimal temperature (IGFBP2, 56 °C; IGFBP3, 63 °C; housekeeping genes, 55 °C) for 1 min, 72 °C for 30 s and repeated for 40 cycles followed by 95 °C for 1 min (1 cycle) and 55 °C for 1 min (1 cycle). Primer efficiencies were greater than 92%. Primers for five housekeeping genes were evaluated for use by the geNorm program (Ghent University Hospital Center for Molecular Genetics, Ghent, Belgium) and GAPDH and cyclophyllin found to be acceptable primer pairs for use as a control. Negative controls lacking cDNA or RT were also utilized. Results for IGFBP2 and IGFBP3 were analyzed using BioRad Excel gene expression macro and the  $\Delta\Delta$ CT data graphed and utilized for statistical analysis.

### 2.4. IGFBP protease activity assay

Media BP protease activity was assessed by Western blot and changes in recombinant human IGFBP-3 band intensity. To measure the activity of media protease on IGFBP-3, 500 ng recombinant hIGFBP-3 was added to media derived from MDBK cells treated for 24 h with TNF- $\alpha$  (100 nM) and/or 1  $\mu$ M forskolin). Different media formulations were tested to compare the low versus high BSA (heated at 60 °C, 15 min) content with and without protease inhibitor cocktail (10  $\mu$ l/ml, Protease inhibitor cocktail for use with mammalian cell extracts, Sigma, St. Louis, MO). The hIGFBP-3 was allowed to incubate in the media for 30 min (room temperature,



22 °C) at which point aliquots of the reaction mixture were added to loading buffer under nonreducing conditions and heated to 95 °C for 5 min. Anti-human IGFBP-3 was previously determined to not recognize bovine IGFBP-3. Proteins were separated on 4–20% acrylamide gradient gels (Invitrogen), transferred to nitrocellulose, probed with primary antibody and images resolved by standard chemiluminescence detection on film and quantification of resolved band densities.

### 2.5. Statistical evaluation

Data were statistically evaluated as an analysis of variance using a mixed linear model approach based on the Proc Mixed Procedure [32] incorporating well-within-plate effects in the random error term. Differences between treatments were significant where  $P < 0.05$ .

## 3. Results

The effects of confluence and associated morphology on the ability of TNF- $\alpha$  to affect the release of IGFBP-2 into the media and modulate metabolic activity are presented in Fig. 1. The top panel (A) demonstrates that if MDBK cells are maintained at confluence for 3–4 days without further splitting, these cultures form domes suggesting the establishment of tight borders and the initiation of a unidirectional water flux and the lifting of the cell patches from the plastic. Further indications of cytokine effects on physiological processes in these cells was evident where dome formation was impaired in the presence of TNF- $\alpha$  at either  $10^{-7}$  or  $10^{-9}$  M, though trypan blue exclusion indicated no TNF- $\alpha$ -related cytotoxic effect on cell death (data not shown). In the bottom panel (B; left side), IGFBP-2 was used as a marker for the effect of confluence state on cell functionality as regards binding protein production. IGFBP-2 band intensities were affected by the state of confluence of the cells ( $P < 0.002$ ). Basal levels of IGFBP-2 were four-fold higher in cultures attaining 90% confluence (subconfluent) as compared to domed confluent cells. Interestingly, the addition of TNF- $\alpha$  significantly decreased IGFBP-2 content in media from subconfluent cells ( $P < 0.03$ ) but was without effect in the domed cells.

Underscoring the necessity for uniformity in the state of confluence in evaluating the effects of cytokines on MDBK cellular metabolism, urea production was also affected by the state of confluence (Fig. 1B; right side). Mean media concentration of urea produced from arginine by MDBK cells from cultures with evidence of dome formation was overall 77% lower than that measured by MDBK cells with subconfluent (90% confluent

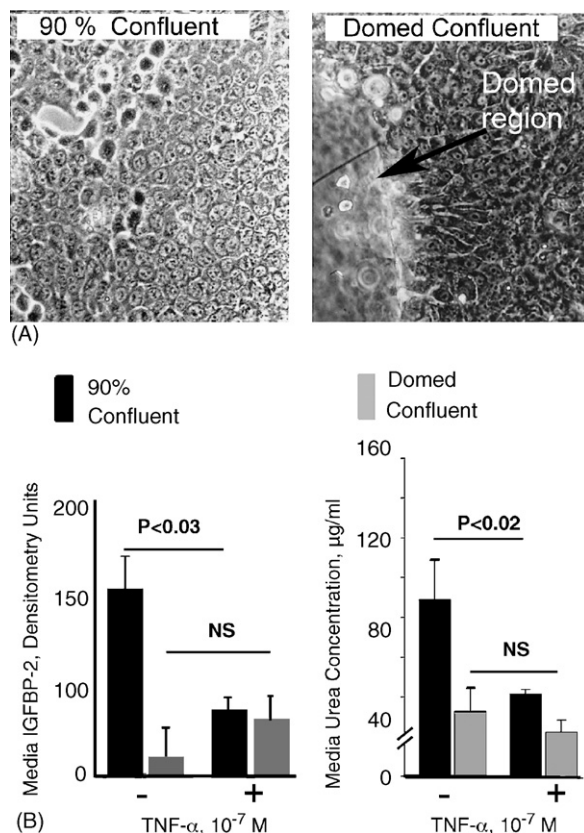


Fig. 1. Effects of state of confluence of cultured MDBK cells on the IGFBP-2 response to TNF- $\alpha$ . The top panel shows examples of subconfluent and confluent-domed cell morphologies. The bottom panel summarizes data on media IGFBP-2 content (Left side) and urea production (right side) from arginine of MDBK cells both confluent and ~90% confluent. IGFBP-2 content was significantly increased in cells of subconfluent status over that measured in cells confluent with doming present. Media urea concentrations from MDBK cells recently attaining confluence were significantly increased over those measured in cells confluent with doming present. The binding protein responsiveness to TNF was significantly affected by culture density. Data represent mean ( $\pm$ S.E.M.) densitometry units for IGFBP-2 Western blots of media from four wells per treatment in replicated experiments.

but not domed) status ( $P < 0.001$ ). Furthermore, while the addition of TNF- $\alpha$  at  $10^{-9}$  M had no significant effect on media concentrations of urea in confluent cultures, media concentrations of urea in subconfluent cells were highly responsive to the effects of TNF- $\alpha$ . TNF- $\alpha$  caused a 44% decrease ( $P < 0.02$ ) in media urea concentrations. Collectively, the data illustrate that the responsiveness of MDBK cells to cytokines are dependent on the stage of development. Thus the interpretation of the effects are greatly influenced by their morphological density characteristics and functionality.

Because MDBK cells were significantly more responsive to stimuli when used at subconfluence, the further

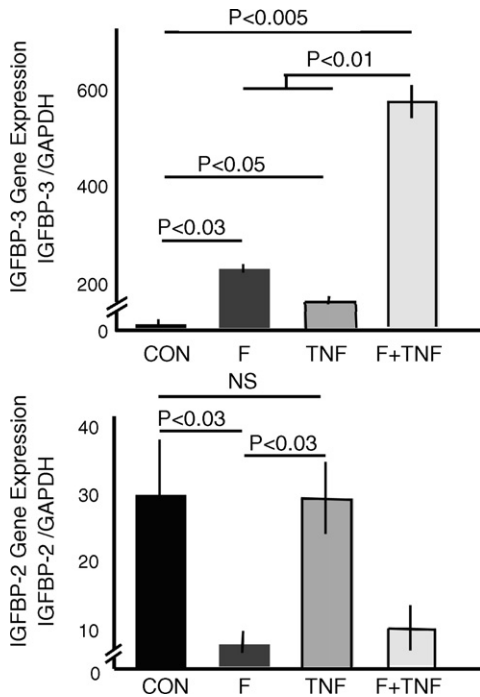


Fig. 2. Effects of forskolin (1.0  $\mu$ M) and TNF- $\alpha$  (100 nM) on gene expression of IGFBP-2 and IGFBP-3 in MDBK cells. Gene expression was normalized to the relative effect and content of GAPDH as a housekeeping gene.

effects of TNF- $\alpha$  to affect gene expression and IGFBP release into media was characterized at this  $\sim 90\%$  density state. The effects of TNF- $\alpha$  on IGFBP-2 and -3 gene expression as normalized against GAPDH in cells under basal and forskolin-stimulated states are illustrated in Fig. 2. IGFBP-3 (top panel) was minimally detectable in MDBK cells under basal conditions, but increased in the presence of both forskolin and TNF- $\alpha$ . TNF- $\alpha$  in the presence of forskolin resulted in an augmented IGFBP-3 gene expression approximately four-fold higher than forskolin or the addition of TNF- $\alpha$  alone ( $P < 0.005$ ). In contrast to IGFBP-3 expression, IGFBP-2 gene expression (bottom panel) was evident under basal conditions and decreased by 73% ( $P < 0.01$ ) by forskolin treatment and was not affected by TNF- $\alpha$  added to culture media.

The effects of TNF and forskolin on IGFBP-3 and -2 peptide media content is presented in Fig. 3. In the top panel, the relative ligand blot band patterns illustrate the differential impact of forskolin and TNF- $\alpha$  on IGFBP-2 and IGFBP-3 released into culture medium by recent confluent MDBK cells. In the bottom panel, the quantitative assessment of mean band intensities indicated that under the culture conditions used (including BSA in the media at 100  $\mu$ g/ml), IGFBP-3 was not present in culture medium but was upregulated and released into

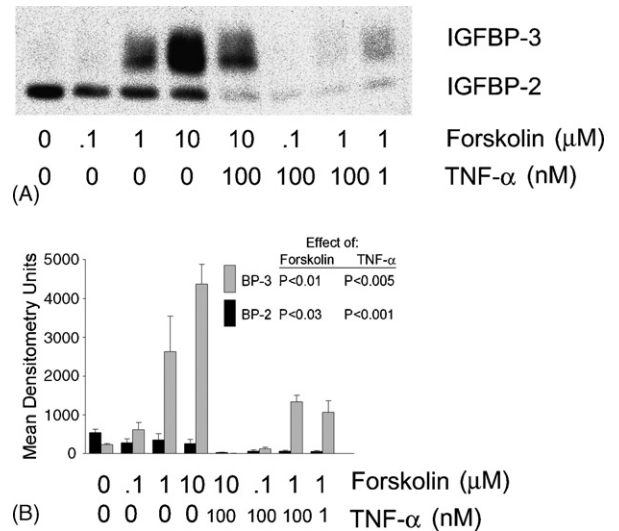


Fig. 3. Effects of TNF- $\alpha$  and forskolin on the MDBK cell culture media content of IGFBP-2 and IGFBP-3 under low (100  $\mu$ g/ml) media protein (BSA) content. (Top panel)  $^{125}$ I-IGF-1 ligand blot demonstrated changes in blot band density for IGFBP-2 and -3. (Bottom panel) Forskolin increased IGFBP-3 media content in a dose related manner whereas IGFBP-2 was decreased in the presence of forskolin. TNF- $\alpha$  further decreased IGFBP-2 content at both TNF- $\alpha$  concentrations and TNF- $\alpha$  similarly blocked the release into media of IGFBP-3. Data represent the mean densitometry units of three to four culture wells representative of three replicated experiments of the respective binding proteins as derived from the scanned autoradiographs.

the medium in a dose-dependent manner by forskolin ( $P < 0.01$ ). IGFBP-2, present under basal culture conditions, was decreased ( $P < 0.05$ ) in the presence of any concentration of forskolin. TNF- $\alpha$  ( $10^{-7}$  M) caused a decrease in forskolin-mediated IGFBP-3 release ( $-53$ ,  $-95$  and  $-99\%$  at 10, 1 and 0.1 M forskolin, respectively ( $P < 0.002$ )). TNF further decreased IGFBP-2 ( $-78$ ,  $-84$  and  $-90\%$  at 10, 1 and 0.1 M forskolin, respectively). In the presence of 1  $\mu$ M forskolin TNF- $\alpha$  at  $10^{-9}$  M decreased media IGFBP-3 and -2 by 68 and 85%, respectively ( $P < 0.02$ ).

The obvious relative lack of continuity and agreement between the gene expression data and media binding protein content was further explored to determine whether there was degradation of the expressed binding protein occurring as a function of cytokine treatment. We tested this hypothesis by altering the potential impact of protease activity in the media. The changes included the addition of bovine albumin to a higher concentration and the use of a protease inhibitor cocktail. Using the described culture conditions, we observed significantly different media binding protein patterns as affected by TNF- $\alpha$  and forskolin depending on whether extra protein and/or protease inhibitors were in the media. As can be

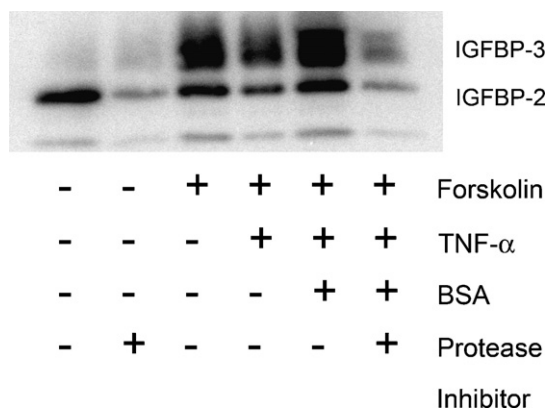


Fig. 4. Effects of forskolin (1.0  $\mu$ M) and TNF- $\alpha$  (100 nM) on the MDBK cell culture media content of IGFBP-2 and IGFBP-2 under high (1 mg/ml) media protein (BSA) content and/or protease inhibitor cocktail.

seen in Fig. 4, IGFBP-3 was again minimally detectable in MDBK media, increased by inclusion of forskolin and decreased by TNF- $\alpha$ . However, if the media content of BSA was increased to 1 mg/ml it became evident that both TNF- $\alpha$  and forskolin were capable of increasing media IGFBP-3 content, consistent with the gene expression data. Interestingly, there was a significant effect of the protease inhibitor cocktail to lower the media content of IGFBP-3 and this was attributed to a potential deleterious effect on the health of the cultured MDBK cells wherein more than 30% of the cells were observed to be damaged or detached from the culture wells shortly after the cocktail was added to the media. This effect on the cells was similarly observed in regard to the IGFBP-2 band pattern intensities where protease inhibitor alone caused an (artifactual) decrease in detectable and measurable IGFBP-2 in media. Again consistent with the gene expression data, we observed no effect of TNF- $\alpha$  on the media content of IGFBP-2. Collectively these data indicate that the major effect of TNF- $\alpha$  was to stimulate IGFBP-3 production in MDBK cells with little if any effect on IGFBP-2. What was very evident was that TNF- $\alpha$  also caused an increase in apparent protease activity that, if left unchecked by the addition of sufficient competitive protein such as BSA, caused a rapid destruction of measurable IGFBPs in culture media.

To further test the protease capacity of the conditioned media, we added a recombinant human IGFBP-3 to TNF- and forskolin-conditioned media, incubated the mixture and resolved the residual hIGFBP-3 by Western blot. The data in panel "A" of Fig. 5 clearly indicate that the resulting proteolytic activity from both forskolin and TNF- $\alpha$  added to media rapidly destroyed measurable hIGFBP-3 in 30 min in a dose-dependent manner

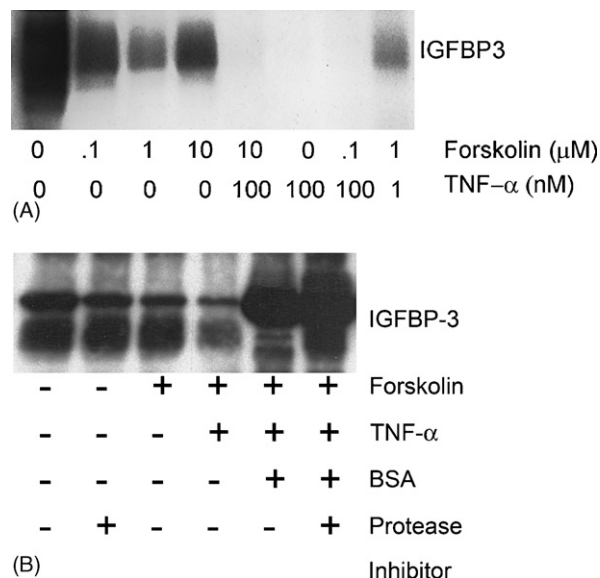


Fig. 5. IGFBP-3 protease assay testing the effects of TNF (100 nM) and forskolin (1  $\mu$ M) on the degradation of recombinant human IGFBP-3 added to conditioned MDBK media. (Top panel) Representative Western blot of hIGFBP-3 after incubation at 37  $^{\circ}$ C in media from MDBK cells containing low BSA. (Bottom panel) Effects of high BSA and protease inhibitor cocktail to preserve the integrity of hIGFBP-3 added to TNF- $\alpha$ - and forskolin media from MDBK cells.

under conditions of low levels of competing media protein. In contrast, when protease inhibitors or additional BSA were added to the media after biological response modifier treatment, the content of measurable IGFBP-3 was largely preserved. Collectively these data indicate that both TNF- $\alpha$  and forskolin cause an increase in media content of a protease with high activity towards IGFBP-3.

#### 4. Discussion

IGFBPs functionally modulate not only the biological half-life of insulin-like growth factors in the circulation, but also the capacity for these growth factors to reach cells across tissues and access the required IGF receptors [8]. Most cells secrete several forms of IGF binding proteins, which are presumed to shape the microenvironment of interactions of the IGFs with the target cells [1,8,12,33,34]. The MDBK cell line previously has been shown to secrete IGFBP-2 under basal conditions, but can also be stimulated via cAMP-associated (i.e., forskolin) pathways to secrete IGFBP-3 [28]. Few investigations have specifically sought to define the effects of proinflammatory cytokine stimuli on the cellular generation and release of IGF binding proteins. The present study has provided the first evidence to suggest that target

cells might respond to immune proinflammatory signals by modifying not only effects on binding protein gene function but also through the elaboration of proteases that impact on the integrity of the binding proteins. The fact that these observations were repeatable as conducted in two different laboratories indicates the continuity of the observed responses. The development of a cytokine-directed variation in the release of key binding proteins during proinflammatory stress reflects the needed capacity of cells and organs to differentiate between and apply selective attributes of the insulin-like growth factors that could span the spectrum of effects ranging from anti-apoptotic functions, tissue repair, modulation of antioxidative capacity to nutrient-specific directed metabolism [3,6,8,16,22,23]. What still remains to be determined is the temporal relationship between the change in IGFBP gene function and the elaboration of the proteolytic events. Indeed, there may be a pattern to the elaboration of several different proteolytic enzymes especially *in vivo*, but this was not tested in these experiments.

The morphological state of the cells affected the capacity to affect binding protein responses to cytokine treatments. The argument can be constructed to imply that the responsiveness of cells to shape their microenvironment as impacted by proinflammatory stimuli might be significantly related to the variation of metabolic capacity inherent in the stage of the cell cycle. In the model employed, we concluded that TNF- $\alpha$  decreased urea production from arginine consistent with effects on the arginase component of the urea cycle [35,36]. The critical feature of this component of the model validation was the fact that at this stage of sub- or early confluence, cells were metabolically active and more sensitive to the effects of the cytokine than were the older cells. In retrospect, the data are consistent with the concept that the proteolytic activity of the confluent-domed cells may have been depressed as compared to that observed in the cultures at approximately 90% confluence, at least as based on the relative capacity to measure the presence of IGFBP-2 and -3.

Using a synchronized MDBK model, we recently demonstrated that the sensitivity of cells to apoptotic stimuli varied with the stage of the cell cycle [37]. In addition, both metabolic activity and proliferation state (cell cycle stage) were significant as regards the development of caspase-mediated, ubiquitination-dependent apoptosis. MDBK cells were least affected by stimuli during the relatively quiescent G<sub>0</sub> state. Similarly, where dome formation was present, cells were most likely arrested in G<sub>0</sub> due to density-dependent contact inhibition. In the present study, where forskolin, a cAMP-mimetic, increased IGFBP-3 in a dose-related manner,

TNF- $\alpha$  attenuated this response significantly, however, only under conditions that prevented released protease from destroying the binding proteins. In this regard, the data from Fig. 3 can also be interpreted to suggest that the elaboration of proteases from the MDBK cells by TNF- $\alpha$  also is a dose-dependent activity. At the same time that TNF- $\alpha$  is increasing gene expression of IGFBP-3 and theoretically therefore also IGFBP-3 protein, it also is stimulating the release of protease. The release of protease therefore hindered the detection of the IGFBP-3 by destroying it.

At the present time there are several classes of metalloproteases that have been implicated as IGFBP (-3) proteases. These include ADAM-12, plasminogen and MMP-7 and their relative change in activity in plasma and tissues during several physiological states (pregnancy, infection, hormonal status) has suggested a regulatory role to further refine IGF-1 action [26,38–40]. The characterization of the types of proteases responsible for the observed effects on the IGF binding proteins was beyond the scope of the present study. The identification of these as produced by MDBK cells under the conditions studied clearly indicates that a very dynamic relationship exists in the production of IGF binding proteins as well as proteases that can interact in a yet-undefined temporal pattern *in vivo* to impact cell regulation by IGF-1. Whereas TNF- $\alpha$  had a clear effect to increase the gene expression of IGFBP-3, there was no evidence of an effect of TNF- $\alpha$  on IGFBP-2 gene expression. However, the cytokine-associated increase in protease release into media from the MDBK cells affected both IGFBP-2 and -3 proteins. The data are consistent with and extend previously observed effects of TNF- $\alpha$  as an inflammatory cytokine to impact the IGF-somatotropic axis during proinflammatory stress [11,24,41,42] through the potential for shaping very localized access of IGF-1 to tissues through the induction and/or destruction of IGFBPs.

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